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Duplex Scorpion primers in SNP analysis and FRET applications

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ABSTRACT

Scorpions are fluorogenic PCR primers with a probe element attached at the 5′-end via a PCR stopper. They are used in real-time amplicon-specific detection of PCR products in homogeneous solution. Two different formats are possible, the 'stem–loop' format and the 'duplex' format. In both cases the probing mechanism is intramolecular. We have shown that duplex Scorpions are efficient probes in real-time PCR. They give a greater fluorescent signal than stem–loop Scorpions due to the vastly increased separation between fluorophore and quencher in the active form. We have demonstrated their use in allelic discrimination at the W1282X locus of the ABCC7 gene and shown that they can be used in assays where fluorescence resonance energy transfer is required.

INTRODUCTION

Scorpion primers are new diagnostic tools for the specific detection of PCR products in real-time (1,2). The basic elements of Scorpions in all formats are: (i) a PCR primer; (ii) a PCR stopper to prevent PCR read-through of the probe element; (iii) a specific probe sequence; and (iv) a fluorescence detection system containing at least one fluorophore and quencher. After PCR extension of the Scorpion primer, the resultant amplicon contains a sequence that is complementary to the probe, which is rendered single-stranded during the denaturation stage of each PCR cycle. On cooling, the probe is free to bind to this complementary sequence, producing an increase in fluorescence, as the quencher is no longer in the vicinity of the fluorophore. The PCR stopper prevents undesirable read-through of the probe by *Taq* DNA polymerase. This would lead to displacement of the quencher and an increase in fluorescence, even in cases where a non-specific PCR product, such as a primer dimer, is formed.

Scorpions technology can be used in allelic discrimination (1,2) and is effective in SNP genotyping (3). In this application the fluorescence is monitored above the T_m of the mismatch probe–target duplex and below the T_m of the fully complementary probe–target duplex. Under these conditions the mismatched probe re-associates with the quencher element to become non-fluorescent, whereas the hybridised wild-type probe is separated from the quencher element and is fluorescent. The intramolecular probing mechanism of Scorpions offers significant advantages over other genotyping systems such as Taqman®™ (4), molecular beacons (5) and hybridisation probes (6) that all rely on bimolecular probing. Unlike Taqman®™ probes, Scorpions do not depend upon enzymic cleavage and, therefore, rapid PCR cycling is possible (2).

Previously, attention has been focused on the single-oligonucleotide 'stem–loop' Scorpion format (1,2,7). In the present study we focus on the two-oligonucleotide 'duplex' format. The mode of action of a stem–loop Scorpion is shown in Figure 1A. The probe sequence is held in a hairpin loop conformation by complementary stem sequences on the 5′ and 3′ sides of the probe, placing the fluorophore in the proximity of the quencher so that collisional quenching occurs. In the duplex format (Fig. 1B) the probe element, which has a fluorophore attached at its 5′-end, is annealed to a complementary oligonucleotide bearing a quencher at the 3′-end. Otherwise the mechanism of action is essentially the same as in the stem–loop format. The intramolecular probe–target interaction, which is the most important feature of the Scorpions system, is maintained in both formats. This results in a very fast and reliable detection system. We report the use of duplex Scorpions in allelic discrimination at the W1282X locus of the ABCC7 gene (8,9), we compare them to stem–loop Scorpions and demonstrate the use of FRET duplex Scorpions.

MATERIALS AND METHODS

Oligonucleotide synthesis

All oligonucleotides were synthesised on an ABI 394 DNA synthesiser by automated solid-phase methods using β-cyanoethyl phosphoramidites. Non-standard monomers (Fig. 2) were prepared in the Oswel laboratory and synthetic details will be published elsewhere. All monomers except ROX were added as phosphoramidites during oligonucleotide synthesis. ROX NHS ester (1 mg in 60 µl DMSO) was added post-synthetically to the 5′-aminohexyl-functionalised Scorpion oligonucleotide (0.2 µmol synthesis) in 0.5 M bicarbonate buffer at pH 9.0 (120 µl) and the resultant solution set aside overnight at room temperature. Oligonucleotides were purified

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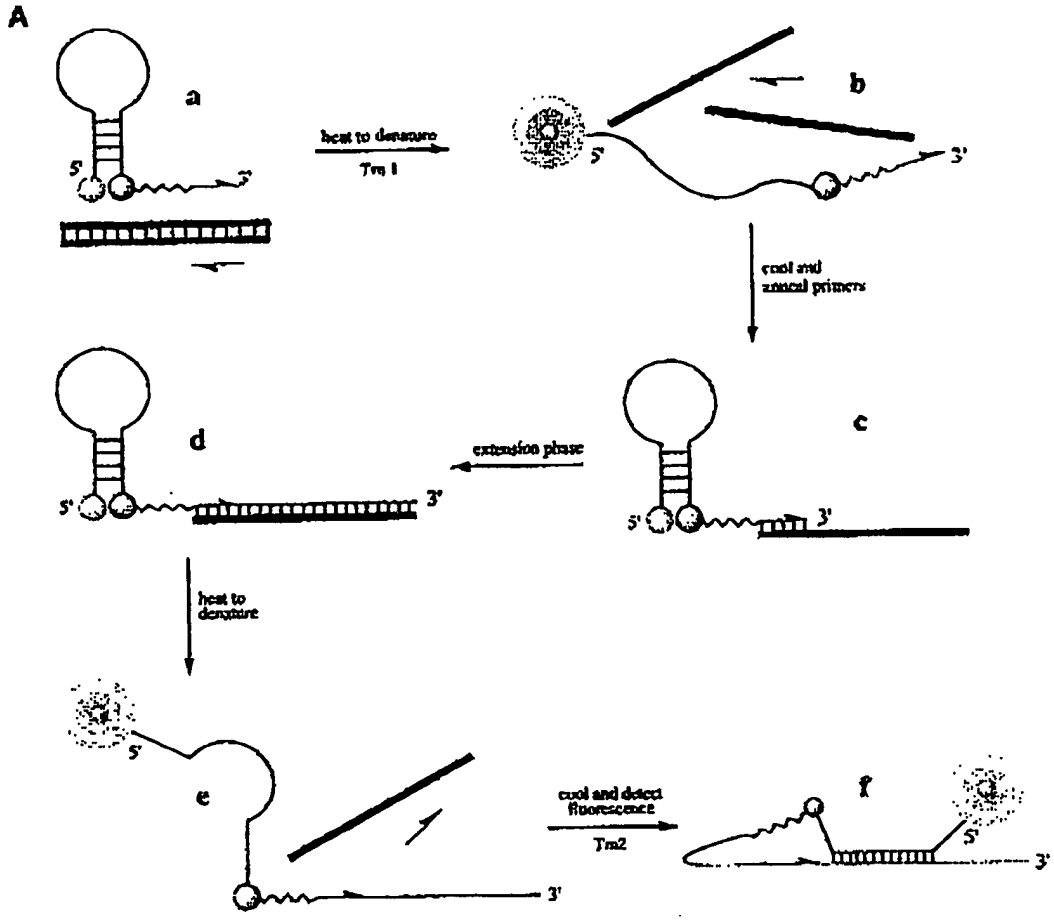

by reversed-phase HPLC on a C8 (octyl) column, eluting with a gradient of acetonitrile in ammonium acetate buffer (10).

Real-time PCR

Human genomic DNA samples, NA 11472 (wild-type) and NA 1723 (W1282X heterozygote), were purchased from Coriell. The samples were stored in 1% bovine serum albumin (BSA) at a concentration of 5 ng/µl. Sequence data for the ABCC7 locus W1282X were obtained from GenBank (11) (see Table 1 for accession numbers and mismatches). Primer sequences were designed using Oligo 4.0 software (National Biosciences Inc., Plymouth, MN) and were placed close to the mutation site to give amplicons of ~100–200 bases. The Scorpion probe sequence was attached to the primer that is closer to the site of mutation. Scorpion folding was evaluated using the *mfold* programme (European mfold server: http://bibiserv.techfak.uni-bielefeld.de/mfold/; M. Zuker, Rensselaer Polytechnic Institute, NY), using the thermodynamic parameters established by SantaLucia (12,13). The design of duplex Scorpions was adapted from the stem–loop version previously evaluated on the W1282X locus (2). All PCR reactions were carried out on a Roche LightCycler. Each reaction was made up from 1 µl (5 ng/µl) of DNA (wild-type, heterozygote, homozygote mutant or negative control), 1 µl of 10× buffer [Advanced Biotechnologies Buffer IV: 200 mM $(NH_4)_2SO_4$, 750 mM Tris–HCl, 0.1% Tween], 1 µl of a 5 µM solution of Scorpion Primer, 1 µl of a 25 µM solution of quencher oligonucleotide when required, 1 µl of 5 µM solution of lower unlabelled primer, 1 µl of a 2 mM solution of dNTPs (each of the four nucleotides), 250 ng/µl of BSA, 0.1 µl (0.5 U) of BioTaq polymerase (Bioline), 1.6 µl of a 25 mM solution of $MgCl_2$ and water to a final volume of 10 µl. DNA was replaced with sterile water for the negative controls. For the SYBR Gold reactions, 1 µl of a 1/1000 dilution of SYBR Gold™ stock solution (Molecular Probes) was added to the PCR reaction. SYBR Gold tests indicated that all DNA samples, either from homo- or heterozygotes, amplified with the same efficiency and there was no measurable PCR inhibition by duplex Scorpions.

The cycling conditions were: initial denaturation for 3 min, 80–100 cycles of 95°C for 0 s, 49°C (annealing temperature) for 0 s and monitoring at 53°C for 3 s. A single fluorescence measurement was made in each cycle during the monitoring

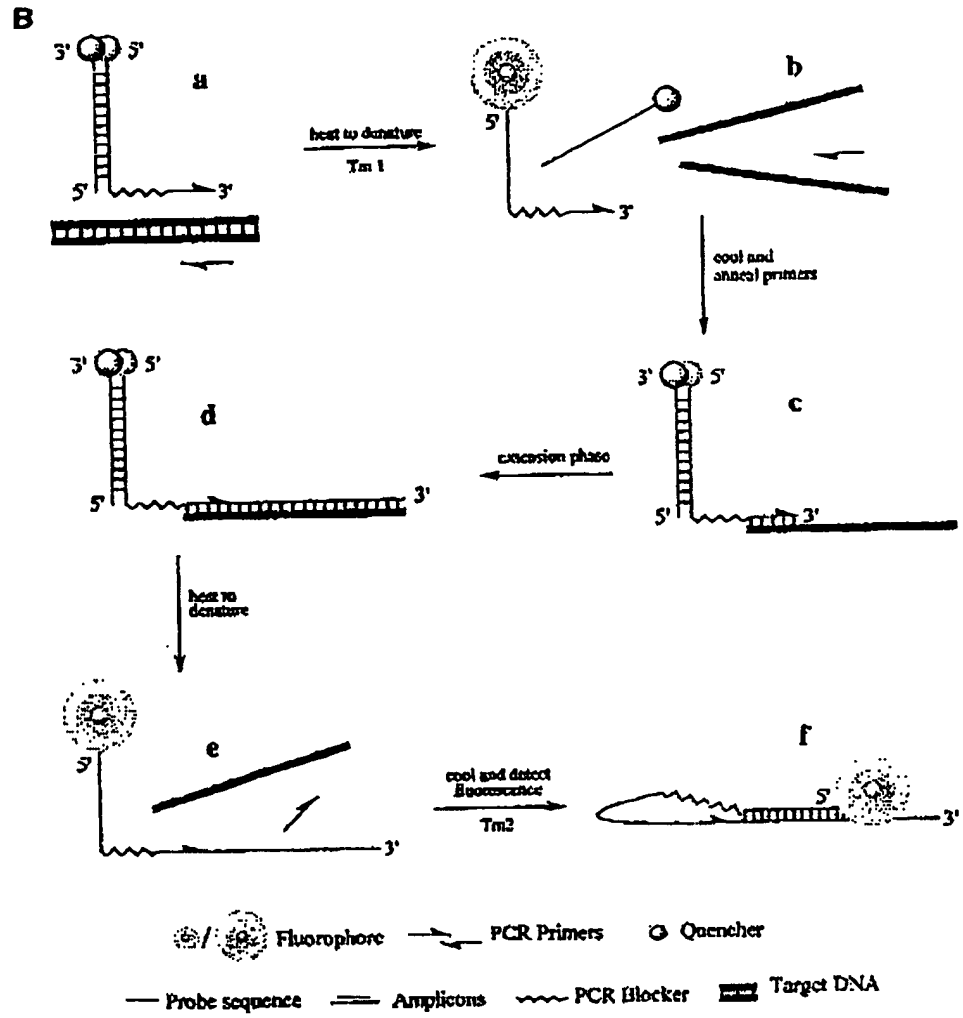

Figure 1. (A) (Previous page) The stem–loop Scorpion format in PCR. On cooling, after PCR primer extension, the probe element binds to its complement on the same strand and fluorescence is observed (structure f). (B) The duplex Scorpion format. The unimolecular probing mechanism is basically the same as in the stem–loop format. Fluorescence of structure f is measured during PCR. wt, wild-type template; het, heterozygote; mut, homozygote mutant; nc, negative control.

Table 1. GenBank accession numbers for locus W1282X

Locus	GenBank accession no.	Mutation site	Base change	Probe-target mismatch
W1282X	M55127	395	G→A	C-A

step. The amplification reached a plateau before the hundredth cycle in all reactions. The temperature conditions were derived from those previously optimised for the stem–loop Scorpions primers (2) and were not modified. The fluorescence gains for the LightCycler were set to F1-2 for all reactions. Data were analysed in the arithmetic and proportional modes as recommended in the LightCycler manual (14). All experiments were carried out in triplicate.

Endpoint analysis

After PCR amplification, samples were heated to 95°C then cooled to 30°C. Fluorescence was then measured. Various heating/cooling rates were compared, all gave acceptable results, 2°C/s was found to be optimum.

Fluorescence melting studies

All melting curves were carried out on a Roche LightCycler. Each reaction was made up from 1 µl of 10× buffer [Advanced Biotechnologies Buffer IV: 200 mM $(NH_4)_2SO_4$, 750 mM Tris–HCl, 0.1% Tween], 1 µl of a 5 µM solution of Scorpion

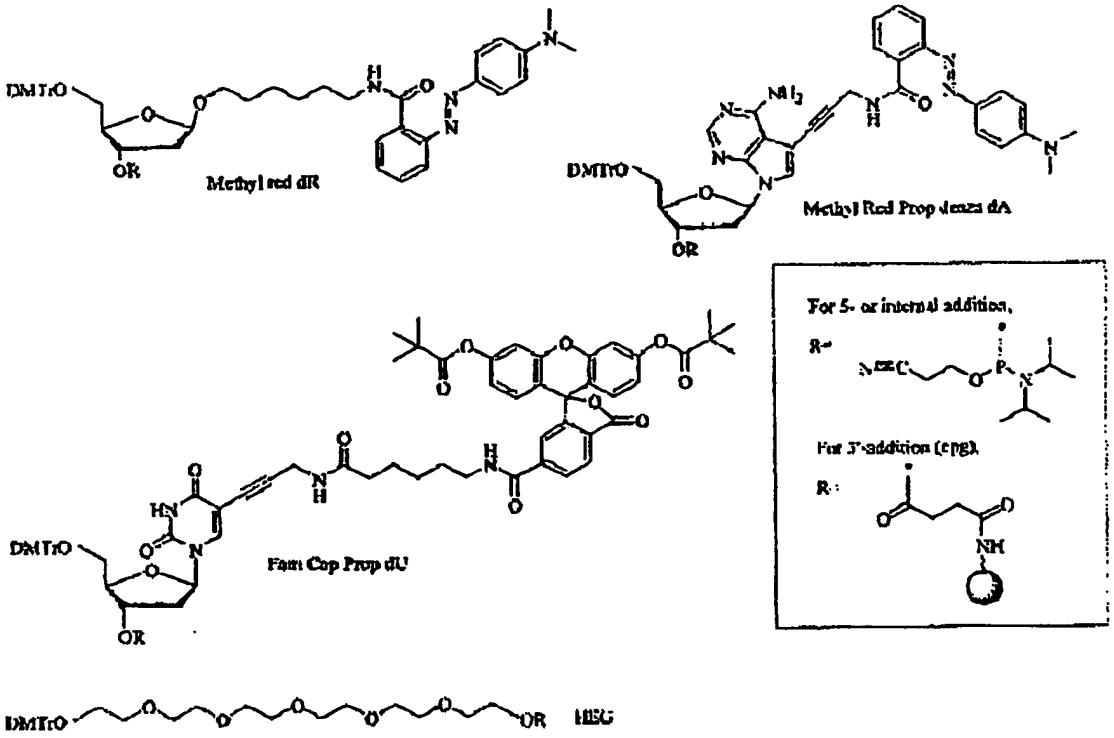

Figure 2. Chemical structures of non-standard monomers.

Table 2. Scorpions and primers sequences

Oligonucleotide name	Code	Oligonucleotide sequence
W1282X reverse primer		GGCTAAGTCCTTTTGCTCAC
Stem–loop FAM Scorpion	W-001	2CCCGCGCCTTTCCTCCACTGTTGCGCGCGGG43ATGGTGTGTCTTGGGATTCA
Duplex FAM Scorpion 1	W-002	2CTTTCCTCCACTGTTGC3ATGGTGTGTCTTGGGATTCA
Quencher oligonucleotide standard	W-003	GCAACAGTGGAGGAAAG4
Quencher oligonucleotide short	W-004	CAGTGGAGGAAAG4
W1282X FRET stem–loop Scorpion	W-005	5CCCGCGCCTTTCCTCCACTGTTGCGACGCGGG76ATGGTGTGTCTTGGGATTCA
W1282X FRET duplex Scorpion 6 base separation	W-006	5CTTTCC6CCACTGTTGC3ATGGTGTGTCTTGGGATTCA
Double quencher oligo 6 base separation	W-007	GCAACAGTGG7GGAAAG4
Internal quencher oligonucleotide	W-008	GCAACAGTGG7GGAAAG8
W1282X FRET duplex Scorpion 11 base separation	W-009	5CTTTCCTCCAC6GTTGC3ATGGTGTGTCTTGGGATTCA
Double quencher oligo 11 base separation	W-010	GCAAC7GTGGAGGAAAG4
W1282X FRET duplex Scorpion 3 base separation	W-011	5CTT6CCTCCACTGTTGC3ATGGTGTGTCTTGGGATTCA
Double quencher oligo 3 base separation	W-012	GCAACAGTGGAGG7AAG4

2 = FAM, 3 = HEG, 4 = Me Red dR, 5 = ROX, 6 = Fam Cap Prop dU, 7 = Me Red Prop deaza dA, 8 = phosphate (see Fig. 2).

Primer, 250 ng/µl of BSA, 1.6 µl of a 25 mM solution of $MgCl_2$ and water to a final volume 20 µl. When required, 1 µl of a 25 µM solution of quencher oligonucleotide was added. Only 10 µl of the reaction mix were used in a single run. The cycling conditions were: initial denaturation for 5 min at 95°C, cooling to 30°C for 0 s, melting from 30 to 95°C with a 0.2°C/s

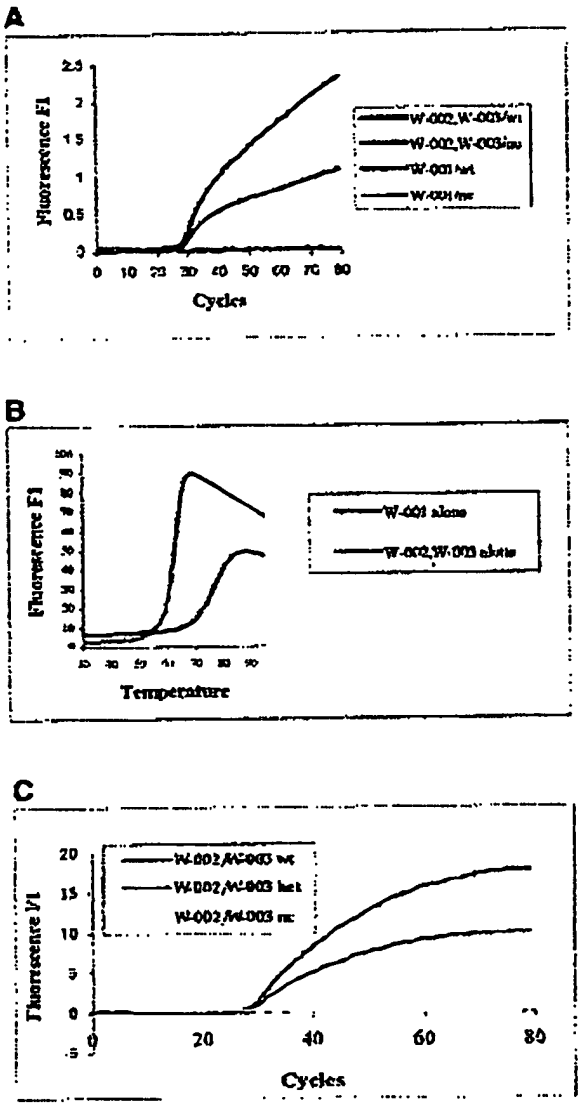

Figure 3. (A) PCR amplification. The fluorescent signal for the duplex Scorpion/quencher pair W-002/W-003 is higher than for the stem–loop Scorpion W-001 (proportional mode). (B) Fluorescence melting curves of stem–loop (W-001) and duplex (W-002/W-003) Scorpion oligonucleotides. (C) Allelic discrimination using the duplex Scorpion/quencher W-002/W-003. Fluorescence for the wild-type template is almost double that of the heterozygote (arithmetic mode). wt, wild-type template; het, heterozygote; nc, negative control.

transition rate and cooling at 40°C for 30 s. Fluorescence was measured continuously during the melting phase.

RESULTS AND DISCUSSION

Comparison between FAM-labelled stem–loop and duplex Scorpions

The FAM-labelled stem–loop Scorpion used in this study (W-001) had been used in previous SNP discrimination studies (2). The equivalent duplex Scorpion (W-002) was derived from W-001 by elimination of the stem and the quencher. A separate quencher oligonucleotide W-003, carrying a methyl red quencher moiety at the 3′-end, and complementary to the probe element of W-002, was used in combination with duplex Scorpion W-002. The chemical structures of the fluorophore and quencher and sequences of the oligonucleotides are shown in Figure 2 and Table 2. Before comparing the two different Scorpions formats, various concentrations of quencher oligonucleotide and duplex Scorpion were evaluated in order to determine the optimum ratio. This was found to be a 5:1 excess of quencher oligonucleotide although the precise ratio was not critical.

In the active conformation of a stem–loop Scorpion, the probe element is bound to the target and fluorescence is produced. The quencher and fluorophore are in the same oligonucleotide and the quencher remains close enough to partially quench the fluorophore by a non-collisional (Förster) mechanism (Fig. 1A, structure f). This must place a limitation on the intensity of fluorescence. This is also a limitation with molecular beacons but not with Taqman™ probes, which are enzymically cleaved during PCR, thus distantly separating the fluorophore from the quencher. In duplex Scorpions, the quencher is in a separate oligonucleotide from the fluorophore, so the two have to be totally separated in the active form and the fluorophore should be completely unquenched (Fig. 1B, structure f). Consequently, the duplex Scorpion system should give a better fluorescent signal than the stem–loop format. It should also give a lower background than Taqman™ probes as quenching in the closed form of duplex Scorpions is predominantly collisional, whereas Taqman probes rely on 'through-space' quenching, the efficiency of which falls off rapidly with increased separation between the fluorophore and quencher.

Indeed, in PCR, the duplex Scorpion/quencher pair W-002/W-003 yielded approximately double the fluorescent signal of the stem–loop Scorpion W-001 (Fig. 3A). The superior signal and lower background is further demonstrated by comparing the fluorescence melting curve of the duplex Scorpion/quencher pair W-002/W-003 with that of the stem–loop Scorpion W-001 (Fig. 3B). This confirms that the increased fluorescent output of the former is due to a fundamental difference in the properties of the two systems and not just a result of differential PCR amplification. The duplex Scorpion also discriminated very well between wild-type and mutant DNA in PCR (Fig. 3C). The fluorescence for the wild-type was about twice the level of heterozygote.

In some cases it may be desirable to use a short quencher oligonucleotide. For example, the same quencher oligo could be used for both the wild-type and mutant Scorpion in a multiplex PCR during allelic discrimination, provided that the quencher oligonucleotide does not cover the SNP site. To investigate the performance of shorter quencher oligos, quencher oligonucleotide (W-004), 4 bases shorter at the 5′-end than the original, was evaluated with duplex Scorpion W-002. This combination gave poor signal to noise ratio (data not shown). The T_m of the W-002/W-004 duplex was then measured on the LightCycler and found to be 56.5°C. Therefore, during the fluorescence monitoring step of PCR, any unextended Scorpions will not be quenched as the W-002/W-004 duplex is not fully formed. This gives rise to the undesirably high fluorescent background. When fluorescence was monitored at

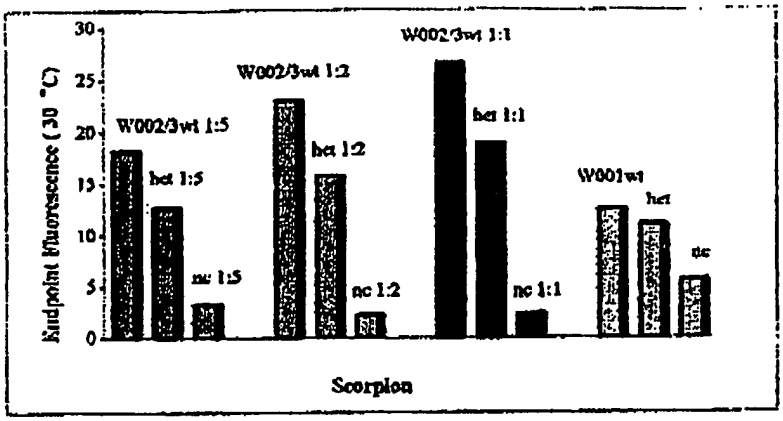

Figure 4. Endpoint analysis. A comparison of stem–loop Scorpion W-001 with duplex Scorpions W-002/W-003 using Scorpion:quencher ratios of 1:1, 1:2 and 1:5. Cooling rate was 2°C/s. In each group the first bar is wild-type (wt), the second heterozygote (het) and the third is the negative control (nc).

43°C, W-002/W-004 gave a signal to noise ratio almost as good as W-002/W-003.

Endpoint analysis

Although real-time PCR detection is an important technique, it requires relatively sophisticated and expensive equipment. A cheaper procedure, which can be performed on a fluorescent plate reader, is to measure the fluorescent signal at the end of a PCR reaction (endpoint analysis). After the PCR, the reaction mixture is heated to 95°C; rapidly cooled to 30°C to 'trap' the active Scorpion and the fluorescent signal is measured (Fig. 4). The wild-type template generates a stronger fluorescent signal than the heterozygote. Several cooling rates were tried, from 0.5 to 5°C, and all gave similar results. The excellent signal t noise ratio of duplex Scorpions makes them ideal for this application. Duplex Scorpions perform better in this application than stem–loop Scorpions for two reasons: (i) the separation between the fluorophore and the quencher in the active form is much greater; (ii) the active species is formed by u favourable intramolecular hybridisation whereas the quenched species

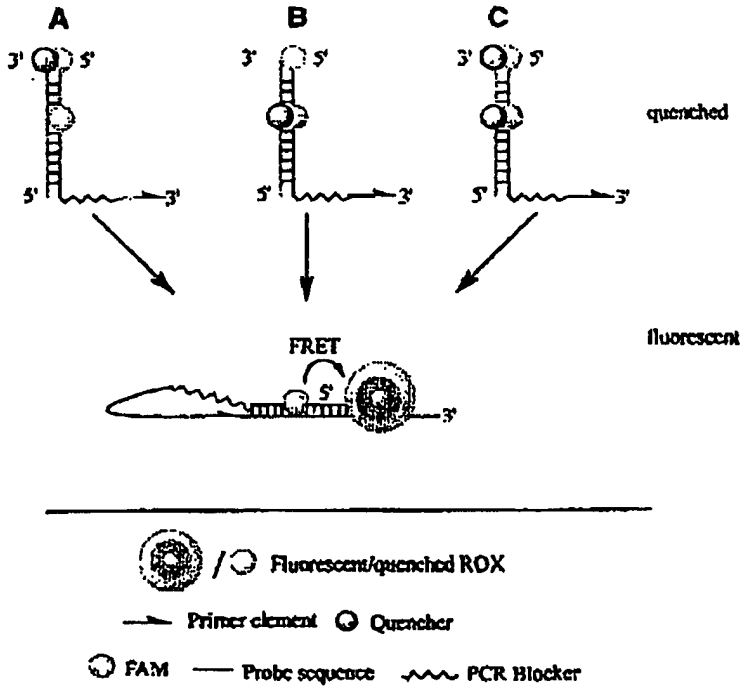

Figure 5. FRET duplex Scorpions with three different versions of the quencher oligonucleotide.

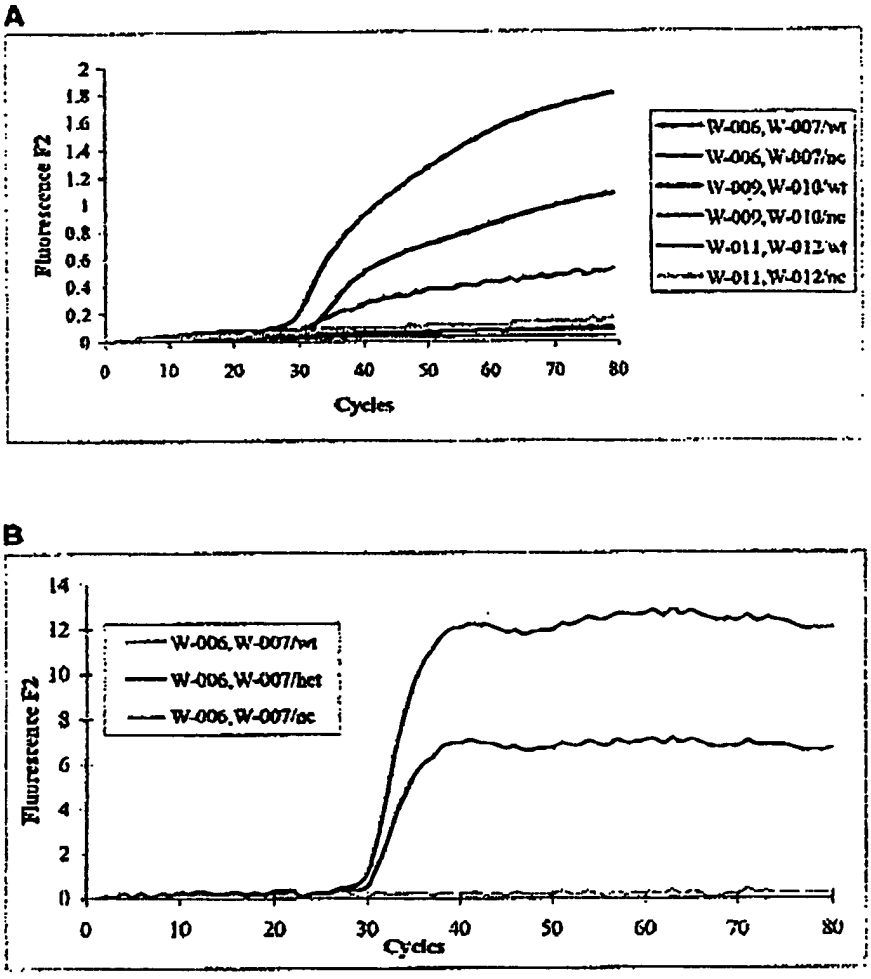

Figure 6. (A) PCR amplification with FRET duplex Scorpions W-006/W-007, W-009/W-010 and W-011/W-012 (proportional mode). ROX fluorescence was monitored. (B) Allelic discrimination with FRET duplex Scorpion W-006/W-007. ROX fluorescence was monitored in channel 2 of the LightCycler (arithmetic mode).

results from a less favoured inter-molecular reaction. Therefore, the fluorescent signal is stable.

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) (15,16) can be used to produce a signal in Channels 2 and 3 of the Roche LightCycler by excitation of fluorescein (FRET donor) and energy transfer to a suitable acceptor dye. The instrument has one excitation source at 488 nm and three channels for detection of the fluorescence emission at 520, 640 and 705 nm. We had previously demonstrated the use of FRET in stem–loop Scorpions (2) and we are now engaged in optimising the technique for the duplex format (Fig. 5). The Scorpions were again evaluated on the W1282X locus and FRET duplex Scorpions were derived from the normal W1282X duplex Scorpion W-002 (Table 2). The FRET Scorpions were labelled at the 5′-terminus with a carboxy X-rhodamine acceptor dye (ROX) that absorbs at 582 nm and emits at 608 nm. A FAM donor fluorophore was incorporated at different internal positions in the probe sequence by means of a modified thymidine, FAM Cap Prop dU.

Three Scorpion/quencher pairs were compared in this study (W-009/W-010, W-006/W-007 and W-011/W-012) with FAM–ROX distances of 11, 6 and 3 bases, respectively. Each quencher oligo has a methyl red dR at the 3′-end opposite t ROX and an internal methyl red dA forming a base pair with the FAM Cap Prop dU moiety in the Scorpion (Fig. 5C). All chemical structures are shown in Figure 2. The increase in fluorescence during PCR was observed in channel 2 of the LightCycler (ROX emission) and colour compensation was used to prevent FAM being detected in this channel. Due t overlap in the emission spectra of fluorophores used on the LightCycler, colour compensation is required to assign emissi n

to one of the detection channels, preventing fluorescence crosstalk (14).

A 6-base inter-fluorophore distance was found to be optimal for efficient FRET to occur (Fig. 6A). An 11-base inter-fluorophore distance gave a less intense fluorescent signal in channel 2 as the efficiency of energy transfer decreased and a 3-base gap gave a much weaker signal due to collisional quenching. In the case of the 6-base inter-fluorophore distance we observed an increase in fluorescence of 12 U for the wild-type template, whereas the increase for heterozygote was –7 U (Fig. 6B).

In the active form of FRET duplex Scorpions, the two fluorophores are in a double-stranded region (probe hybridised to target). At a distance of 6 bp they are on opposite sides of the helix and, therefore, unlikely to partake in undesirable collisional quenching. They are less constrained than in the active form of the stem–loop format where they are in the relatively mobile single strand of the unhybridised stem. In this case a degree of collisional quenching of the acceptor by the donor can be expected, leading to suppression of fluorescence. In order to investigate the physical properties in more detail, we performed fluorescence melting experiments on the three FRET duplex Scorpions W-009/W-010, W-006/W-007 and W-011/W-012 and the FRET stem–loop Scorpion W-005. The stem–loop Scorpion gave a higher background than any of the duplex Scorpions (Fig. 7A). The greatest fluorescence was produced by W-009/W-010 (11-base inter-fluorophore distance) followed by W-006/W-007 (6-base gap), then W-011/W-012 (3-base gap). In duplex Scorpions there seems to be significant quenching of the ROX by the FAM when the two dyes are separated by 6 bases and less quenching when the separation is 11 bases. This result appears to contradict the PCR results in Figure 6A but there is in fact no contradiction. In the melting experiment the ROX fluorescence increases as the quencher oligo dissociates and the Scorpion becomes single stranded. In this form, the flexibility of the DNA strand allows the ROX and FAM to get much closer together than when fluorescence is measured during PCR at which point the probe element is in a duplex (Fig. 5). In terms of separation it is likely that 6 bases in the duplex form is similar in distance to 11 bases in the single-stranded form.

An interesting feature of duplex Scorpions is the sharp melting transition observed when the quencher oligo diffuses away from the Scorpion (Figs 3B and 7A). This co-operative dissociation is highly desirable as it liberates the probe element of the Scorpion over a narrow, well-defined temperature range. By determining the T_m of a duplex Scorpion/quencher pair in the PCR buffer (T_m 1 in Fig. 1B), we can ensure that the chosen fluorescence monitoring temperature in the PCR cycle is well below this T_m. Thus, the background fluorescence from unhybridised duplex Scorpion that has not yet been converted to an amplicon will be very low when fluorescence is monitored.

Next we focused our attention on the efficiency of quenching in the FRET duplex Scorpion to determine whether it is absolutely necessary to have two quenchers, one for the FRET donor and one for the acceptor (Fig. 5). Three quencher oligonucleotides were compared for their ability to quench the preferred duplex FRET Scorpion W-006. One had a single methyl red moiety at the 3′-end (Fig. 5A) opposite to ROX in the W-005 FRET Scorpion (W-003), the second had a methyl red within the sequence (W-008) on an adenine base (Fig. 5B)

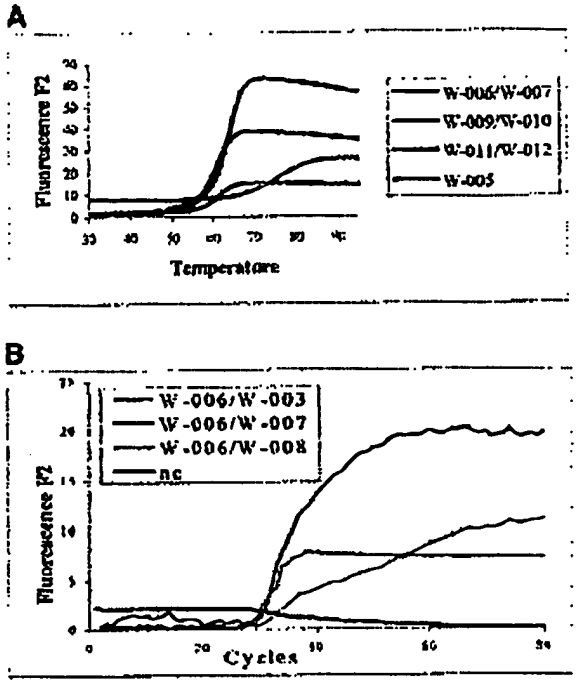

Figure 7. (A) Fluorescence melting curves: ROX emission of FRET duplex Scorpions W-006/W-007, W-009/W-010, W-011/W-012 (6-, 11- and 3-base separation between ROX and FAM) and FRET stem–loop Scorpion W-005. (B) Variations in the quencher oligonucleotide and their effect on the fluorescence output in FRET duplex Scorpions in PCR. Wild-type template was used with duplex Scorpion W-006 (ROX–FAM distance of 6 bases). W-003 has a 3′-terminal methyl red dR, W-008 has an internal methyl red Prop deaza dA and W-007 has both methyl red quenchers. Arithmetic mode. ROX fluorescence measured.

opposite the FAM dU of W-006 and the third oligonucleotide (Fig. 5C) had a methyl red in both positions (W-007). The use of two methyl red moieties in the quencher oligonucleotide improved the signal to background ratio (Fig. 7B). The increase in fluorescence was always greater than the case in which only one methyl red was employed, and good allelic discrimination was achieved (Fig. 6B). On average an increase of 14 U in fluorescence was observed for the wild-type template and the fluorescence for heterozygote samples was about half.

CONCLUSIONS

The duplex Scorpion system comprises of three oligonucleotides: a Scorpion primer, a normal reverse primer and a quencher oligonucleotide. The system can be used in real-time PCR for SNP genotyping and is a suitable format for use on platforms such as the Roche LightCycler when FRET is required to utilise the available channels. Duplex Scorpions have significant advantages over their stem–loop counterparts, producing a more intense fluorescent signal due to the vastly increased separation between fluorophore and quencher in the active form. FRET duplex Scorpions are simpler t synthesise and significantly easier t purify by HPLC than stem–loop

Scorpions, as the fluorescent dye pair and the quencher pair are in different ligonucleotides. Duplex Scorpions are also suitable for use in fluorescent endpoint analysis.

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